Reaction Mechanism of DL-2-Haloacid Dehalogenase from Pseudomonas sp. 113: Hydrolytic Dehalogenation Not Involving Enzyme-Substrate Ester Intermediate (MOLECULAR BIOFUNCTION-Molecular Microbial Science)

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CITATION:

Esaki, Nobuyoshi ...[et al]. Reaction Mechanism of DL-2-Haloacid Dehalogenase from Pseudomonas sp. 113: Hydrolytic Dehalogenation Not Involving Enzyme-Substrate Ester Intermediate (MOLECULAR BIOFUNCTION-Molecular Microbial Science). ICR Annual Report 2000, 6: 46-47

ISSUE DATE:

2000-03

URL:

http://hdl.handle.net/2433/65226

Reaction Mechanism of DL-2-Haloacid Dehalogenase from *Pseudomonas* sp. 113: Hydrolytic Dehalogenation Not Involving Enzyme-Substrate Ester Intermediate

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DL-2-Haloacid dehalogenase from *Pseudomonas* sp. 113 (DL-DEX 113) catalyzes the hydrolytic dehalogenation of D- and L-2-haloalkanoic acids, producing the corresponding L- and D-2-hydroxyalkanoic acids, respectively. L-2-Haloacid dehalogenase, haloalkane dehalogenase, and 4-chlorobenzoyl-CoA dehalogenase, which catalyze the hydrolysis of various organohalogen compounds, have an active site carboxylate group that attacks the substrate carbon atom bound to the halogen atom, leading to the formation of an ester intermediate. This is subsequently hydrolyzed, resulting in the incorporation of an oxygen atom of the solvent water molecule into the carboxylate group of the enzyme. In the present study, we analyzed the reaction mechanism of DL-DEX 113. When a single turnover reaction of DL-DEX 113 was carried out with a large excess of the enzyme in $H_2{}^{18}O$ with either D- or L-2-chloropropionate, the major product was found to be ^{18}O-labeled lactate. After a multiple turnover reaction in $H_2{}^{18}O$, the enzyme was digested with proteases, and the molecular masses of the peptide fragments were measured. No peptide fragments contained ^{18}O. These results indicate that the $H_2{}^{18}O$ of the solvent directly attacks the α-carbon of 2-haloalkanoic acid to displace the halogen atom. This is the first example of an enzymatic hydrolytic dehalogenation that proceeds without formation of an ester intermediate.

Keywords: 2-Haloacid dehalogenase/ Ionspray mass spectrometry/ Reaction mechanism

Halidohydrolases catalyze hydrolytic cleavage of carbon-halogen bonds of various organohalolgen compounds. 2-Haloacid dehalogenases (EC 3.8.1.2), haloacetate dehalogenases (EC 3.8.1.3), haloalkane dehalogenases (EC 3.8.1.5), and 4-chlorobenzoyl-CoA dehalogenases (EC3.8.1.6) are included in this group of enzymes. 2-Haloacid dehalogenases are further classified into the following three groups: 1) L-2-Haloacid dehalogenase (L-DEX) catalyzes hydrolysis of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids. 2) D-2-Haloacid dehalogenase (D-DEX) catalyzes the conversion of D-2-haloalkanoic acids into L-2- hydroxyalkanoic acids. 3) DL-2-Haloacid dehalogenase (DL-DEX) dehalogenates both D- and L-2-haloalkanoic

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Scope of research

Structure and function of biocatalysts, in particular, pyridoxal enzymes and enzymes acting on xenobiotic compounds, are studied to elucidate the dynamic aspects of the fine mechanism for their catalysis in the light of recent advances in gene technology, protein engineering and crystallography. In addition, the metabolism and biofunction of selenium and some other trace elements are investigated. Development and application of new biomolecular functions of microorganisms are also studied to open the door to new fields of biotechnology. For example, molecular structures and functions of psychrophilic enzymes and their application are under investigation.

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acids to produce the corresponding L- and D-2-hydroxyalkanoic acids, respectively. DL-DEX is similar to racemases and epimerases in that it acts on the chiral center of both D- and L-enantiomers indiscriminately. However, this enzyme is unique in that it catalyzes a chemical conversion on the chiral centers of both enantiomers.

Reactions catalyzed by L-DEX from *Pseudomonas* sp. YL (L-DEX YL), haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, and 4-chlorobenzoyl-CoA dehalogenases from *Pseudomonas* sp. strain CBS3 and *Arthrobacter* sp. 4-CB1 have been shown to proceed as in Fig. 1 (A) [1]. These mechanisms resemble each other in that the reactions proceed through ester intermediates formed from catalytic acidic amino acid residues of the enzymes and their substrates. The ester intermediates are subsequently hydrolyzed releasing the products and restoring the carboxylate groups of the enzymes.

DL-DEXs have been purified from *Pseudomonas* sp. 113 (DL-DEX 113), *Pseudomonas putida* PP3, and *Rhizobium* sp. However, none of the reaction mechanisms of these DL-DEXs has been studied, and it has remained unknown whether the reaction mechanism of DL-DEX is similar to that of other halidohydrolases. We previously determined the primary structure of DL-DEX113, and found that it is similar to that of D-DEX from *Pseudomonas putida* AJ1 [2]. We also showed that DL-DEX 113 has a single and common catalytic site for both D- and L-enantiomers based on a site-directed mutagenesis experiment and kinetic analysis [2]. In the present study, we analyzed the reaction mechanism of DL-DEX 113 by means of ^{18}O incorporation experiments [3].

We conducted the single turnover reaction of DL-DEX 113 in $H_2{}^{18}O$ with D- or L-2-chloropropionate as a substrate, using an excess amount of the enzyme. We found that a majority of the lactate produced was labeled with ^{18}O. This makes a clear contrast with the results on the L-DEX YL reaction, which proceeds through the mechanism involving an ester intermediate (Fig. 1 (A)). Only 10% of the D-lactate produced from L-2-chloropropionate by L-DEX YL was labeled with ^{18}O. This suggests that in the DL-DEX 113 reaction an oxygen atom of the solvent water was directly incorporated into the product. While supporting the mechanism shown in Fig. 1 (B), this is not compatible with the mechanism in Fig. 1 (A), in which an oxygen atom of the solvent water is first incorporated into the enzyme.

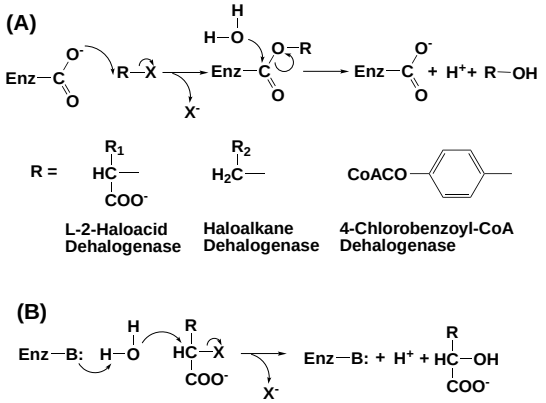

Figure 1. Reaction mechanisms of dehalogenases

A multiple turnover reaction of DL-DEX 113 was carried out in $H_2{}^{18}O$ with D- or L-2-chloropropionate as a substrate. After completion of the reaction, the enzyme was digested with TPCK-treated trypsin, and the resulting peptide fragments were separated on a reversed phase column interfaced with an ionspray mass spectrometer as a detector. If the reaction proceeds through the mechanism in Fig. 1 (B), ^{18}O should not be detected in the proteolytic fragments. The molecular masses of all peptides were virtually indistinguishable from the predicted ones whether the reaction was conducted with D- or L-2-chloropropionate. Since peptides containing amino acid residues 1, 106-107, 135-142, 181-183, 229-238, 250-254, 284-285, and 299-300 were not found in the trypsin-digested sample, we also analyzed lysyl endopeptidase-digested enzyme by the same method. Peptides 120-142, 232-285, and 299-306 were identified, and their molecular masses were virtually indistinguishable from the predicted ones.

In the above experiment, the molecular masses of the peptides containing amino acid residues 1, 106-107, 181-183 and 229-231 could not be measured. Therefore, we could not exclude the possibility that ^{18}O was incorporated into Asp or Glu in these peptides. However, among these peptides, only peptide 181-183 contains an acidic residue, Asp181. We replaced Asp181 with Ala, Arg, and Glu by site-directed mutagenesis to clarify whether Asp181 is involved in the catalytic reaction shown in Fig. 1 (A). The activities of these mutant enzymes were similar to that of the wild-type enzyme, indicating that Asp181 is not essential for the catalysis.

All the above results are consistent with the general base mechanism shown in Fig. 1 (B), but not with the mechanism shown in Fig. 1 (A). This applies to the dehalogenations of both enantiomers of 2-haloalkanoic acids because the results obtained for both enantiomers were virtually the same. We previously reported that DL-DEX 113 has a single and common catalytic site for both L- and D-enantiomers based on a site-directed mutagenesis experiment and kinetic analysis. This conclusion is supported by our present data showing that the enzymatic dehalogenations of both enantiomers proceed through the same mechanism as shown in Fig. 1 (B). DL-DEX 113 is unique in that its reaction does not involve the formation of an ester intermediate. Since D-DEX from *Pseudomonas putida* AJ1 shows sequence similarity with DL-DEX 113, the reaction of D-DEX probably proceeds through the mechanism shown in Fig. 1 (B). Although DL-DEX and L-DEX can catalyze the same reaction (hydrolysis of L-2-haloalkanoic acids), our present data clearly show that the reaction mechanisms of DL-DEX and L-DEX are completely different from each other.

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